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Article

Extracts from Amazonian plants have inhibitory activity against tyrosinase: an *in vitro* evaluation

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Dermatological disorders related to pigmentation result in tenuous hyper or hypopigmentation Cosmetic and pharmaceutical products containing depigmenting substances are used in the treatment of patients who have high pigmentation disorders, such as melasma or chloasma, post-inflammatory hyperpigmentation, senile lentigo and ephelides. Skin lightening agents are not yet totally effective or safe and therefore intensive research for the discovery of new agents is continuous. Enzyme inhibitors involved in melanogenesis, such as tyrosinase, have been discovered in Asian countries, including those isolated from plant extracts. The Brazilian flora has the highest species diversity in the world, and the chemical, pharmacological and cosmetic potential for the discovery of new skin whitening agents is in proportion with this biodiversity. For these reasons, 25 aqueous and 24 organic extracts obtained from 19 plants native to the Amazon rain forest and to the Atlantic forest, belonging to 11 different families, were evaluated as tyrosinase inhibitors. Nine out of 49 extracts showed inhibitory activity in the screening process. The 50% inhibitory activity (IA_{50}) was calculated, revealing that the most active extracts were the organic extracts from the leaves and stem of *Ruprechtia* sp. (IA_{50} 33.76 $mg.mL^{-1}$) and the organic extract from the aerial organs of *Rapanea parviflora* (IA_{50} 64.19 $mg.mL^{-1}$).

Uniterms: Tyrosinase. Melanin. Plant extract/*in vitro* evaluation. Skin lightening agents. Amazonian plants/evaluation/dermatological use.

Problemas dermatológicos relacionados com a pigmentação resultam em hiperpigmentações ou hipopigmentação cutâneas. Produtos cosméticos e farmacêuticos com atividade despigmentante são utilizados para o tratamento de pacientes que apresentam distúrbios de hiperpigmentação, tais como melasma ou cloasma, hiperpigmentação pós-inflamatória, lentigem senil e efélides. Os despigmentantes atualmente utilizados não são totalmente eficazes ou seguros, razão pela qual há intensa pesquisa, principalmente em países asiáticos, com a finalidade de se obter novos agentes com esta ação, em especial inibidores de enzimas envolvidas na melanogênese, como a tirosinase. Considerando-se que algumas substâncias obtidas de plantas apresentam essa atividade, a flora brasileira constitui-se uma fonte potencial de obtenção de novos despigmentantes. Por essa razão, 25 extratos aquosos e 24 orgânicos obtidos de 19 plantas da Floresta Amazônica e Mata Atlântica, provenientes de 11 diferentes famílias, foram avaliados quanto à atividade de inibição da tirosinase. Do total de 49 extratos testados, 9 mostraram atividade. Os valores de concentração da atividade inibitória 50% (AI 50%), foram calculados e o mais ativo foi o extrato orgânico das folhas e caule de *Ruprechtia* sp. (AI_{50} 33,76 $mg.mL^{-1}$) seguido do extrato orgânico dos órgãos aéreos de *Rapanea parviflora* (AI_{50} 64,19 $mg.mL^{-1}$).

Unitermos: Tirosinase. Melanina. Extrato de plantas/avaliação *in vitro*. Despigmentantes. Plantas amazônicas/avaliação/uso dermatológico.

INTRODUCTION

For centuries, humans have tried to artificially modify the color of their skin, both by using whitening or darkening products. Such attitudes were related to philosophy, beliefs, religion and mainly to vanity (Shevlin, 1974). Over-exposure of the skin to ultraviolet light is related to human ostentation, but it can also be related to pigmenting problems or even to skin cancer (Podahaisky

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et al., 2002). As the world population ages it becomes even more important to treat pigment disorders (Benech, 2002). Severe anomalies may also hamper an individual in social relationships (Grimes, 1999).

Both hyper and hypopigmentation are treated with cosmetic or pharmaceutical products containing depigmenting substances, which are synthetic or of natural origin. Pigmentation disorders can also be treated with mechanical peeling allied to chemical substances or treatments with laser rays (Grimes, 1999).

The skin produces a complex mixture of enzymes for protection from the constant exposition to environmental oxidative stress (Podahaisky *et al.*, 2002), but in some situations this is insufficient to prevent disorders from appearing. However, the fact that drugs can be absorbed by the skin (Thorsteinsson *et al.*, 1999) aids in the development of cosmetic preparations to treat skin pigmentation or depigmentation, particularly drugs whose active substance is a natural product.

One of the most effective depigmenting substances is hydroquinone, which inhibits the synthesis of melanin. Enzyme inhibition is one of the mechanisms involved in melanogenesis (Lee, Choi, 1998).

Many screening studies are reported in the literature, with emphasis on those products made with tyrosinase inhibitors from African plants (Baurin *et al*., 2002; Kubo, Hori, 1999b, Momtaz *et al*., 2008), Bolivia (Kubo *et al.*, 1995), China (Iida *et al.*, 1995; Masamoto *et al.*, 1980; Miao *et al.*, 1997), Japan (No *et al.*, 1999), Bangladesh (Khanom *et al.*, 2000) and others. Positive results in relation to activity were obtained and some authors continued this work by isolating active substances.

Considering that current therapies have shown less than satisfactory results in the treatment of various dermatological disorders such as melasma, post-inflammatory or senile lentigo and ephelides, and that the side effects of the therapy include high cytotoxicity and mutagenicity, poor skin penetration and low stability of formulations (Grimes, 1999; Nerva *et al.*, 2003), new drugs with enzyme inhibitory activity are needed(Su, 1999).

Satisfactory results obtained from screening studies previously conducted by other groups, reporting the identification of inhibitory activity of melanogenesis by plant extracts, allied with the fact that Brazilian biodiversity corresponds to 20% of the world's biodiversity (Suffredini *et al.*, 2004) justify the current project of screening Brazilian plant extracts such as tyrosinase inhibitors *in vitro,* so that agents may be found and eventually used in cosmetic and pharmaceutical products.

MATERIAL AND METHODS

Plant extracts

The plant material listed in Table I was collected from *igapó*, *terra firme* and Atlantic forests, according to a chemosystematic approach. Vouchers were deposited in the Herbarium UNIP, where they were identified. After collection, the material was ground up and 24h-macerated with a mixture of dichloromethane and methanol (1:1), followed by 24h-maceration with Milli-Q water. The organic extracts were evaporated under reduced pressure (Büchi) and the aqueous extracts were lyophilized (Virtis). All the extracts were stored in a freezer (-27 ºC, Revco) until use (Younes *et al*., 2000).

Tyrosinase enzymatic reaction assay

The assay was realized with modifications (Khanom, 2000; Kobayashi *et al*., 1995), such that 10 mL of the solution made with 120 $U.mL^{-1}$ (1st screening) or 480 $U.mL^{-1}$ (2nd screening) of tyrosinase obtained from mushrooms (Sigma) were added to 96-well microplates, as were 70mL of pH 6.8 buffer solution and 60 mL of the plant extract solutions (water or DMSO 50% in water, were used as vehicles). A negative control was used, as was a positive control made with a solution of kojic acid. To this mixture, 70 µL of L-tyrosine (Sigma) were added, completing the final volume up to 210 µL. The absorbance was taken at 490nm in a microplate spectrophotometer reader (Biotek) in the beginning of the reaction, as the time zero reading. Microplates were incubated at (30±1) ºC for 120 minutes in the first screening, and for 60 minutes in the second screening. Optical densities were registered on a computer coupled to the spectrophotometer reader. Inhibitory activity of 50% was obtained according to the formula

$$IA\ (\%) = [((C-T_0)-(S-T_0))/(C-T_0)] \times 100$$

where IA = inhibitory activity, C= control absorbance at 490 nm, S= test absorbance at 490 nm and T_0= time zero, and each parameter was a mean of 8 measures.

The percentage of inhibitory activity of tyrosinase obtained from the extracts was compared to that obtained from the kojic acid.

Tyrosinase inhibitory activity by plant extracts

The first screening was performed as previously described, using tyrosinase at 120 $U.mL^{-1}$. The final

concentration in the assay was 80 µg/mL (µg.ml^{-1}) for the extracts, and 5 µg.mL^{-1} for kojic acid, used as a positive control. Readings were taken 120 minutes after the addition of the extract or kojic acid. The active extracts were submitted to a second screening, whose enzyme concentration was 480 U.mL^{-1}.

Determination of the inhibitory activity at 50%, of kojic acid using tyrosinase at concentrations of 120 U.mL^{-1} and 480 U.mL^{-1}

The test was performed as first described. Tyrosinase was diluted to 120 U.mL^{-1} and 480 U.mL^{-1}, in the first and second screening, respectively. Kojic acid concentrations used in the test were 5.0, 4.0, 3.0, 2.5, 1.25, 0.625 and 0.3125 µg.mL^{-1} in the first screening, and 10.0, 5.0, 2.5, 1.25 and 0.625 µg.mL^{-1} in the second screening.

Determination of the inhibitory activity at 50%, of the active extracts

The test was executed as first described, substituting the kojic acid by plant extracts, whose concentrations were 80.0, 40.0, 20.0 and 10.0 µg.mL^{-1}.

TABLE I - List of Amazonian Rain Forest plant extracts tested against tyrosinase

Extract*	Species	**Family**	**Plant organ**	**Collection number**
01/O 02/A	*Calophyllum brasiliense* Cambess	Clusiaceae	Stem	PS187
03/O 04/A	*Ruprechtia* sp.	Polygonaceae	Leaves and Stem	PS387
05/O 06/A	*Mabea nitida* Spruce ex Benth	Euphorbiaceae	Leaves and Stem	PS81
07/O 08/A	*Piranhea trifoliata* Baill.	Euphorbiaceae	Leaves	PS88
09/A	*Aparisthmium cordatum* (A. Juss) Baill.	Euphorbiaceae	Aerial organs	PS393
10/O 11/A	*Piranhea trifoliata* Baill.	Euphorbiaceae	Fruits	PS88
12/O 13/A	*Calophyllum brasiliense* Cambess	Clusiaceae	Fruits	PS187
14/O 15/A	*Mabea nitida* Spruce ex Benth.	Euphorbiaceae	Fruits	PS81
16/O 17/A	*Malouetia tamaquarina* (Aubl.) A. DC.	Apocynaceae	Aerial organs	IBS10
18/O 19/A	*Calophyllum brasiliense* Cambess	Clusiaceae	Leaves	PS187
20/O 21/A	*Duguetia uniflora* (DC.) Mart.	Annonaceae	Stem bark	PS357
22/O 23/A	*Guatteria riparia* R. E. Fr.	Annonaceae	Stem	PS115
24/O 25/A	*Crudia amazonica* Spruce ex Benth.	Leguminosae Caesalpinioidae	Stem	PS90
26/A	*Guatteria riparia* R. E. Fr.	Annonaceae	Leaves	PS115
27/O 28/A	*Couma utilis* (Mart.) Mill. Arg.	Apocynaceae	Leaves	AAO3336
29/O 30/A	*Macrolobium acaciifolium* (Benth.) Benth.	Leguminosae Caesalpinoidae	Fruits	PS137

TABLE I - List of Amazonian Rain Forest plant extracts tested against tyrosinase (cont.)

Extract*	Species	Family	Plant organ	Collection number
31/O 32/A	*Hevea microphylla* Ule	Euphorbiaceae	Stem	PS196
33/O 34/A	*Taralea oppositifolia* Aubl.	Leguminosae Faboidae	Leaves	PS108
35/O 36/A	*Taralea oppositifolia* Aubl.	Leguminosae Faboidae	Fruits	PS108
37/O 38/A	*Piranhea trifoliata* Baill.	Euphorbiaceae	Stem	PS88
39/O 40/A	*Triplaris surinamensis* Cham.	Polygonaceae	Stem	AAO3294
41/O 42/A	*Davilla rugosa* Poir.	Dilleniaceae	Aerial organs	AAO3457
43/O 44/A	*Passiflora* sp.	Passifloraceae	Aerial organs	AAO3421
45/O 46/A	*Rapanea parviflora* Aubl. **	Myrsinaceae	Aerial organs	AAO3458
47/O 48/A	*Palicourea grandifolia* (A. DC) Mez	Rubiaceae	Aerial organs	AAO3542
49/O	*Casearia javitensis* Kunth	Flacourtiaceae	Aerial organs	AAO4067

*O = organic extract; A = aqueous extract; ** = Atlantic Forest

RESULTS AND DISCUSSION

IA_{50} of the kojic acid obtained from the reaction with tyrosinase at 120 $U.mL^{-1}$, calculated from the regression line formula generated after 120 minutes of reaction, was 2.99 $\mu g.mL^{-1}$. The IA_{50} of the kojic acid, obtained from regression line formula generated from the reaction with tyrosinase at 480 $U.mL^{-1}$ after 60 minutes was 6.85 $\mu g.mL^{-1}$.

Results obtained in the first screening of plant extracts as inhibitors of tyrosinase are shown in Table II (statistical analysis, T test). The IA_{50} concentrations are shown in Table III.

Skin clarity is an important characteristic, as hyper and hypopigmentation affect the self-esteem of an individual, but fortunately can be treated, or at least minimized. In the last few years the number of whitening products on the market has increased dramatically, but therapies have not always shown satisfactory results (Su, 1999; Tengamnuay *et al.*, 2006), This is principally due to the high toxicity of the whitening substances, such as that observed for hydroquinone (Grimes, 1999). The present observations highlight the need for intensive research on new depigmenting substances. The identification of enzyme inhibitors involved in melanogenesis, such as hydroquinone and kojic acid, is especially important.

Given that plants may contain enzyme inhibitory substances, screening methodologies show greater efficacy in the identification of active substances (Lee *et al.*, 1999), and there are calls to search for active compounds from Brazilian plants, the present screening was delineated in order to evaluate plant extracts obtained from plants chemosystematically related to groups whose phenolic compound production is significant (Iida *et al.*, 1995; Kubo, Hori, 1999a; Kubo, 1995; No, 1999). Plants belonging to the families: Annonaceae, Apocynaceae, Clusiaceae, Dilleniaceae, Euphorbiaceae, Flacourtiaceae, Leguminosae, Myrsinaceae, Passifloraceae, Polygonaceae and Rubiaceae were assayed. Some plants of these families have already been evaluated and have been shown to contain phenolic tyrosine inhibitor compounds such as flavonoids and tannins (Iida *et al.*, 1995; Khanom *et al*., 2000).

The tyrosinase inhibitory effect of 49 aqueous and organic extracts obtained from 19 Brazilian plants belonging to 11 families was evaluated using a spectrophotometric method. Kojic acid was chosen as a positive control because the substance is an effective inhibitor of tyrosinase *in vitro* (Sandoval, 1999).

The concentration of tyrosinase used in the first screening was 120 U.mL^{-1} in order to increase the sensitivity and chances of finding active extracts. As the chemical composition of such extracts is not known, further studies were needed in order to verify the presence of tyrosinase inhibitor substances isolated by bioguided fractionation, and will be discussed below.

Calculations of the extracts' inhibitory activity on melanogenesis were based on the comparison between data obtained from tests with extracts, and from negative controls. The extracts that showed significant inhibitory activity in the 120 minute screening obtained from the first screening (03/0, 07/0, 45/0, 11/A, 50/A, 21/A, 32/A, 34/A and 45/A), and the kojic acid, were submitted to a second screening in order to determine the inhibitory activity (IA%) and IA_{50}, using a concentration of tyrosinase of 480 U.mL^{-1}.

As shown in Table III, all the extracts except 34/A, demonstrated positive inhibitory activities. Nonetheless, the linearity of the curves obtained to calculate IA_{50} were not always significant (R^2> 0.90), as was the case with extracts 45/O, 15/A and 21/A.

The relative comparison of test and control IA_{50} showed that IA_{50} obtained for kojic acid was 6.85µg.mL^{-1} and that the extract 03/O (*Ruprechtia* sp. - stem and leaves - Polygonaceae, IA_{50} 33.76µg.mL^{-1}) was the most potent enzyme inhibitor. Nonetheless, the dose dependent relationship was not as significant (R^2=0.803) as that obtained for extract 45/O (*Rapanea parviflora* - aerial organs - Myrsinaceae, IA_{50} 64.19 µg.mL^{-1}, R^2=0.9935). Both extracts can be considered potential anti-tyrosinase agents based on the IA_{50} and R^2 values found in the present study and on comparison with the values obtained by Khanom *et al*. (2000), where kojic acid yielded IA_{50} = 7.5 µg.mL^{-1},

TABLE II - Tyrosinase reaction in the presence of plant extracts at different concentrations after 60 minutes of exposure

	Extract concentration µg.mL^{-1}													
Extract	Control**		10			20			40			80		
	Abs	SD	Abs	SD	p	Abs	SD	p	**Abs**	**SD**	**p**	**Abs**	**SD**	**p**
03/O	0.5299	0.0139	0.3763	0.0123	<0.0001*	0.2511	0.0142	<0.0001*	0.1684	0.0249	<0.0001*	0.0915	0.0146	<0.0001*
07/O	0.5299	0.0139	0.5008	0.0086	0.0002*	0.5064	0.0268	0.0450*	0.5013	0.0699	0.2754	0.4545	0.0331	<0.0001*
45/O	0.5430	0.0131	0.4838	0.0539	0.0092*	0.4624	0.0211	<0.0001*	0.3583	0.0136	<0.0001*	0.2118	0.0093	<0.0001*
11/A	0.5005	0.0516	0.4439	0.0064	0.0082*	0.4478	0.0128	0.0141*	0.4446	0.0056	0.0087*	0.4606	0.0164	0.0559
15/A	0.5005	0.0516	0.4668	0.0205	0.1081	0.4714	0.0383	0.2211	0.4568	0.0223	0.0452*	0.4194	0.0086	0.0006*
21/A	0.5840	0.0156	0.5680	0.0064	0.0178*	0.5318	0.0095	<0.0001*	0.4659	0.0117	<0.0001*	0.3874	0.0211	<0.0001*
32/A	0.5840	0.0156	0.5799	0.0122	0.5675	0.5673	0.0080	0.0175*	0.5631	0.0236	0.0554	0.5711	0.0337	0.3425
34/A	0.5583	0.0086	0.5633	0.0216	0.5527	0.5656	0.0161	0.2770	0.5628	0.0227	0.6082	0.5673	0.0199	0.2599
44/A	0.5583	0.0086	0.5475	0.0263	0.2882	0.5394	0.0205	0.0306*	0.5468	0.0251	0.2404	0.5660	0.0117	0.1559

p = values for test T; *=significant; Abs = mean absorbance (n=8); SD = Standard deviation; ** = negative control (without extract).

TABLE III - Tyrosinase inhibitory activity of plant extracts.

Extract	IA %*				Equation	R^2	IA_{50}*
	10	20	40	80			
03/O	28.99	52.61	68.22	82.73	y = 0.9282x + 18.664	0.8032	33.76
07/O	5.50	4.40	5.40	14.23	y = 0.1529x + 1.3198	0.8748	318.37
45/O	10.91	14.85	34.02	61.00	y = 0.7559x + 1.4798	0.9935	64.19
11/A	11.31	10.54	11.16	7.97	y = 0.0446x + 6.8585	0.0872	nd
15/A	6.70	5.80	8.70	16.21	y = 0.1764x + 2.1911	0.9077	271.03
21/A	2.74	8.95	20.23	33.67	y = 0.4354x + 0.0568	0.9845	114.70
32/A	0.71	2.87	3.55	2.31	y = 0.0027x + 1.0773	0.3298	nd
34/A	-0.90	-1.32	-0.81	-1.61	y = - 0.0144x – 0.4975	0.5511	nd
44/A	1.93	3.31	2.06	-1.32	y = 0.0293x + 2.0743	0.2537	nd

IA (%) = percentage of inhibitory activity; R^2 = coefficient of linear correlation;
IA_{50} = inhibitory activity at 50%; nd = not determined; *= µg.mL

and the extract obtained from *Glycyrrhyza glabra* had $IA_{50} = 21.2\ \mu g.mL^{-1}$.

CONCLUSIONS

The extracts obtained from the leaves and stem of *Ruprechtia* sp. and from the aerial organs of *Rapanea parviflora* were considered the most active in the present assay. Extracts obtained from the leaves of *Piranhea trifoliata*, fruits of *P. trifoliata*, fruits of *Mabea nitida*, stem bark of *Duguetia uniflora*, stem of *Hevea microphylla*, leaves of *Taralea oppositifolia* and the aerial organs of *Passiflora* sp. were found to exhibit anti-tyrosinase activity. These may be interesting candidates for evaluation in more complex biological system assays such as toxicity *in vitro*, melanocyte cultures and eventually in human *in vivo* assays.

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